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NOTES

Surface Layer Protein EA1 Is Not a Component of *Bacillus anthracis* Spores but Is a Persistent Contaminant in Spore Preparations

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EA1 is an abundant, highly antigenic, surface layer protein of *Bacillus anthracis* vegetative cells. Recent studies indicate that EA1 is also a component of *B. anthracis* spores and a potential marker for spore detection. We show here that EA1 is not a spore component but a persistent contaminant in spore preparations.

Surface layers, or S-layers, are two-dimensional paracrystalline arrays that cover vegetative cells of many prokaryotic organisms (9). Typically, S-layers form by noncovalent, entropy-driven self-assembly of identical protein or glycoprotein subunits. For some species, alternative S-layers and S-layer proteins are present at the cell surface. In the case of *Bacillus anthracis*, the causative agent of anthrax, the S-layer is composed of either extracellular antigen 1 (EA1) or surface array protein (Sap), which is encoded by the chromosomal gene *eag* or *sap*, respectively (3). EA1 is the main S-layer protein produced in *B. anthracis* strains carrying plasmid pXO1, which contains genes necessary to produce anthrax toxins and other virulence-related proteins (4). In fact, EA1 is the most abundant protein and the major cell-associated antigen in these strains (3).

Several recent studies indicate that EA1 is also a component of the *B. anthracis* spore surface and could be used as a species-specific molecular marker for detection of spores (2). Here we investigate these possibilities and discuss the adverse consequences of using EA1 as a marker for *B. anthracis* spores.

Evidence indicating that EA1 is a spore component. Three observations suggested that EA1 was present on spores of *B. anthracis*. First, in a proteomic analysis of spore surface proteins of *B. anthracis*, Lai et al. reported the presence on highly washed spores of a 91,362.5-Da (pI = 5.70) cell surface antigen containing S-layer homology domains (2). They also identified this protein by matrix-assisted laser desorption ionization–time of flight mass spectrometry as protein NP_654830 in the National Center for Biotechnology Information database. Although not stated in this report, the sequence of this 862-amino-acid protein is identical to that of EA1.

Second, from a small fraction of our *B. anthracis* spore preparations, we were able to extract a variable amount of an approximately 100-kDa protein under conditions that solubilized proteins on the spore surface. Most of our spore preparations were devoid of this protein. In all cases, spores were derived from the avirulent Sterne strain ($pXO1^+$ $pXO2^-$; unable to produce vegetative cell capsule), which was grown in Difco sporulation medium at 37°C with shaking for 36 to 48 h (5). Under these culture conditions greater than 95% of the cells sporulate. The spores were washed extensively with water and pelleted through 50% Renografin to remove vegetative cell debris (12). This widely used protocol is generally regarded

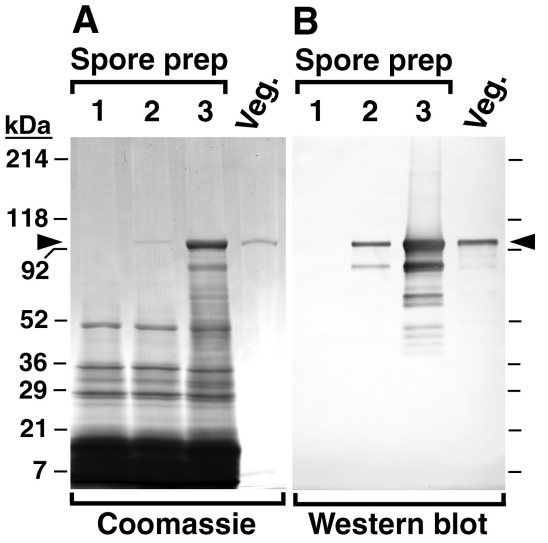

FIG. 1. Variable amounts of the ~100-kDa protein (EA1) were extracted from selected preparations of *B. anthracis* spores. A typical spore preparation (preparation 1) and two preparations with low (preparation 2) and unusually high (preparation 3) amounts of the ~100-kDa protein (EA1) were analyzed. As a control, we also analyzed an extract of *B. anthracis* vegetative cells (Veg.). (A) Proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue. The ~100-kDa protein (EA1) (indicated by the filled arrowhead) was a major protein in spore preparation 3 and one of only two proteins observed in the vegetative cell extract (with 4 μg of protein loaded in the lane). (B) The electrophoretically separated spore and vegetative cell proteins described for panel A were analyzed by Western blotting. Identical results were obtained with either SA26 or M2-V129 as the primary antibody, and only the results with SA26 are shown.

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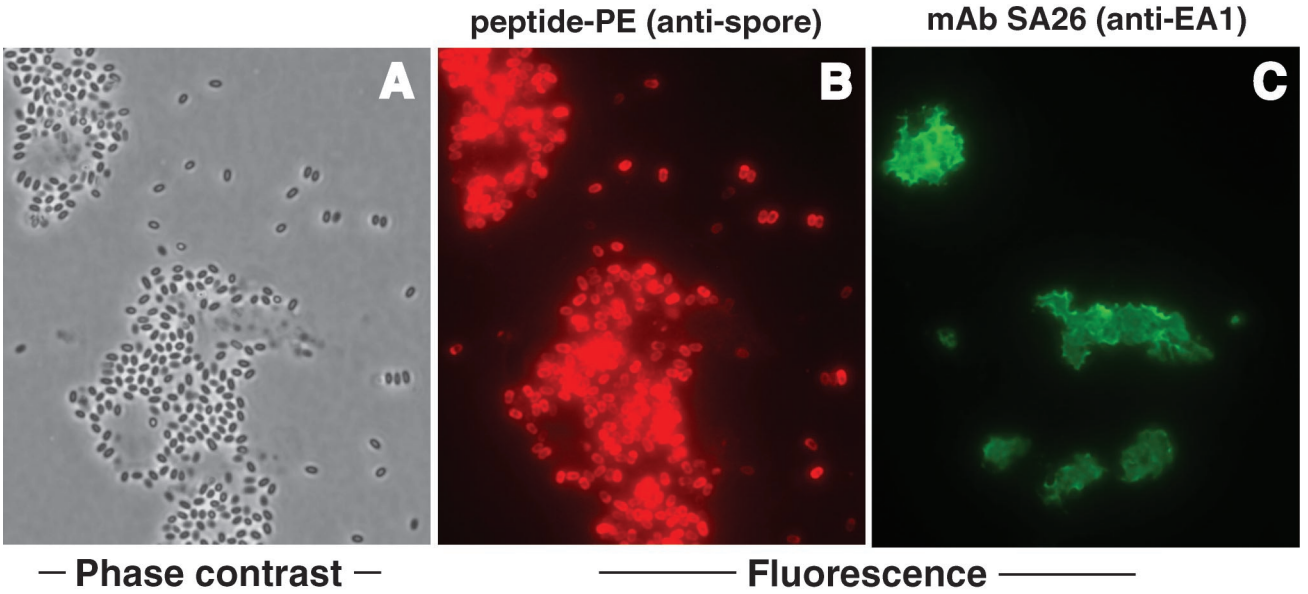

FIG. 2. Binding of an anti-EA1 MAb (SA26) to contaminating material in spore preparations but not to spores. The figure shows spores and contaminating material in spore preparation 3, which contains high levels of EA1. The sample was examined by phase-contrast microscopy (A) and by fluorescence microscopy under conditions that detect the fluorescence of either phycoerythrin (PE) (B) or Alexa 488 (C) (12, 13).

as the most rigorous method for spore purification (1, 5). Spore extracts, which contained primarily proteins of the outermost spore layer (i.e., the exosporium), were prepared by boiling 3 $\times$ 10^8 spores from each preparation for 8 min in 20 μl of sample buffer containing 125 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 10% (vol/vol) 2-mercaptoethanol, 1 mM dithiothreitol, 0.05% bromophenol blue, and 10% (vol/vol) glycerol (10). The samples were then spun at 10,600 $\times$ *g* for 3 min, and the proteins in the supernatants were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on a 4 to 15% polyacrylamide gradient gel (Ready Gels; Bio-Rad Laboratories). The occasional presence of variable amounts of the ~100-kDa protein band is depicted in Fig. 1A, which shows a Coomassie blue-stained gel of electrophoretically separated proteins that had been extracted from three spore preparations.

To identify the ~100-kDa protein, we excised the band from the gel and determined its amino-terminal amino acid sequence by automated Edman degradation. The resulting sequence was AGKSFPDVPA, which corresponds uniquely to the amino terminus of EA1 after removal of a 29-amino-acid signal peptide (3, 7). In addition, we demonstrated that extracts of vegetative cells of *B. anthracis* (Sterne) contained a highly abundant protein that comigrated with the ~100-kDa protein variably extracted from spores (Fig. 1A). This vegetative cell protein was previously identified as EA1 (3, 4).

Third, commercial mouse monoclonal antibodies (MAbs) advertised as recognizing 92- to 94-kDa *B. anthracis* spore proteins were recently made available. The MAbs are designated SA26 (or ab2281) and M2-V129 and are sold by Novus Biologicals, Inc. (supplied by Abcam), and OEM Concepts, respectively. Reportedly, these MAbs do not cross-react with *B. anthracis* vegetative cells or with spores of *B. globigii*, *B. subtilis*, or *B. cereus* (the latter being the species most phylogenetically similar to *B. anthracis* [6]). The size of the spore antigen(s) recognized by MAbs SA26 and M2-V129 and the highly antigenic character of EA1 prompted us to investigate the possibility that these MAbs were reacting with EA1 present in spore extracts. Therefore, we analyzed by Western blotting the separated proteins of the spore and vegetative cell extracts shown in Fig. 1A. After SDS-PAGE, the proteins were transferred to duplicate nitrocellulose membranes and treated as described in the manual for the Bio-Rad Immun-Blot assay kit. The membranes were probed separately with SA26 and M2-V129 (each at 5 μg/ml), and bound MAb was detected with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) at 2 μg/ml (Bio-Rad Laboratories). Identical results were obtained with each MAb, which clearly show that both MAbs react with EA1 in extracts prepared from spores and vegetative cells (Fig. 1B). Cross-reactive bands that migrated faster than 100 kDa in the gel presumably were proteolytic fragments of EA1. In addition, we demonstrated that SA26 and M2-V129 reacted with intact vegetative cells of the Sterne strain of *B. anthracis* (data not shown), contrary to the claims of the commercial suppliers of the MAbs.

Evidence that EA1 is not a spore component but a contaminant in spore preparations. To determine if EA1 was in fact a component of the spore surface, we used fluorescence microscopy to examine directly the binding of (anti-EA1 MAb) SA26 to *B. anthracis* spores. Using all three spore preparations analyzed in Fig. 1, we dried 10^6 spores (in 10 μl) of each onto separate poly-L-lysine-coated glass microscope slides (Sigma). The immobilized spores were then treated with 1% bovine serum albumin to block nonspecific binding sites and washed three times with 1 ml of cold (4°C) phosphate-buffered saline (PBS) (8) containing 0.5% Tween 20 (Sigma). A 30-μl drop of SA26 (5 μg/ml in PBS) was placed on each spore sample, and the slides were incubated for 1 h at 4°C in a humid chamber.

The spores were washed as described above. We then placed on each spore sample a 30-μl drop containing (in PBS) Alexa 488-labeled goat anti-mouse IgG (2 μg/ml) (Molecular Probes) and 400 nM peptide (ATYPLPIR)-phycoerythrin conjugate that binds specifically to *B. anthracis* spores (13). (The peptide ligand was included only to help visualize spores [see below].) The samples were incubated and washed as described above and examined by phase-contrast and fluorescence microscopy.

Examination of spores in preparation 3, which contained high levels of EA1, was most revealing (Fig. 2). Many bright spores were observed by phase-contrast microscopy (Fig. 2A), and all spores were fluorescently (red) labeled with the peptide-phycoerythrin ligand (Fig. 2B). In contrast, no spores were fluorescently (green) labeled with SA26 (as detected with Alexa-labeled anti-mouse IgG) (Fig. 2C). Instead, SA26 bound to irregularly shaped particulate material typically much larger than a spore. This material was present in large amounts in spore preparation 3 and in low amounts in spore preparation 2 and was undetectable in spore preparation 1 (data not shown). These results and the observed reactivity with SA26 indicated that the particulate material contained large aggregates of EA1, presumably derived from vegetative cells. The particulate material could also be observed in liquid suspension by phase-contrast microscopy, although not as well as when dried. In suspension, the particulate material formed smaller clusters and was essentially devoid of vegetative cells (data not shown). Note that, in control experiments, we confirmed that the reactivity of SA26 and its detection with anti-mouse IgG were the same in the presence and in the absence of the peptide-phycoerythrin conjugate (data not shown).

Determining the source of EA1 in spore preparations. Our results clearly demonstrated that EA1 was present in our spore preparations as a contaminant. To understand how this contamination occurred and could be avoided, we monitored EA1 levels at four stages of the standard procedure for the preparation of highly purified spores (1, 5). EA1 levels were measured after spores were harvested from 30 ml of culture, after the spores had been washed with 30 ml of cold (4°C) water each day for 3 days, after the spores were sedimented through 50% Renografin, and after the Renografin-purified spores had been washed three times with 10 ml of cold water. A sample containing 3×10^8 spores was removed from each fraction and treated as if to extract spore surface proteins as described above. Proteins in each sample were separated by SDS-PAGE on a 4 to 15% polyacrylamide gradient gel and visualized by staining with Coomassie brilliant blue (Fig. 3). The results show that a large amount of EA1, apparently contained in vegetative cell debris, was collected with the spores upon initial harvesting by centrifugation (lane 1). Extensive washing of the spores removed a negligible amount of EA1 (lane 2). However, sedimentation through 50% Renografin removed all but trace amounts of EA1 (lane 3). This small amount of EA1 was removed by additional washing (lane 4). These results indicate that contamination of spore preparations with EA1 occurs (occasionally) because of the large amount of this protein that is collected with the spores and the persistence of this protein throughout most steps of the purification protocol. In addition, the results suggest that a high level of EA1 contamination is most likely due to a problem with the Renografin purification step.

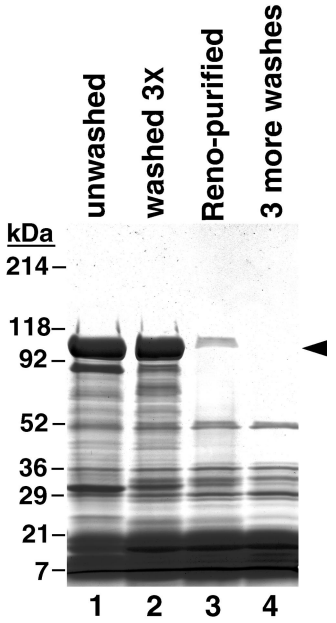

FIG. 3. Levels of contaminating EA1 at selected stages of purification of *B. anthracis* spores. For each stage, a sample containing the same number of spores was treated to extract spore surface proteins, and these proteins were separated by SDS-PAGE and visualized by Coomassie blue staining. EA1 levels were measured after spores were harvested (lane 1), after spores were washed three times (lane 2), after spores were sedimented through 50% Renografin (lane 3), and after spores were washed three more times (lane 4). The position of EA1 in the gel is marked with a filled arrowhead.

Concluding remarks. Our results demonstrate that EA1 is not present on the surface of *B. anthracis* spores. The presence of this protein in spore preparations is due to contamination. This result is consistent with the observation that inactivation of the *eag* gene has no apparent effect on sporulation or spore structure (11). However, the presence of contaminating EA1 in spore preparations is a potentially important problem for the detection of pathogenic spores of *B. anthracis*. Many detectors of *B. anthracis* spores rely on antispore antibodies, especially MAbs, as sensors. These antibodies are raised against spores that are typically not highly purified. Thus, these preparations are likely to be contaminated with highly antigenic EA1, and antibodies raised against these preparations are likely to react (primarily) with EA1. The incorporation of anti-EA1 antibodies into spore detectors would lead to the detection of a nonpathogenic contaminant and not spores. This situation could result in the failure to detect highly purified spores or to accurately estimate spore number. Both failures could have catastrophic consequences.

Protein sequencing was performed in the UAB Cancer Center Shared Facility for Protein Analysis.

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